

Cell Signaling during Cold, Drought, and Salt Stress

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INTRODUCTION

Low temperature, drought, and high salinity are common stress conditions that adversely affect plant growth and crop production. The cellular and molecular responses of plants to environmental stress have been studied intensively (Thomashow, 1999; Hasegawa et al., 2000). Understanding the mechanisms by which plants perceive environmental signals and transmit the signals to cellular machinery to activate adaptive responses is of fundamental importance to biology. Knowledge about stress signal transduction is also vital for continued development of rational breeding and transgenic strategies to improve stress tolerance in crops. In this review, we first consider common characteristics of stress signal transduction in plants, and then examine some recent studies on the functional analysis of signaling components. Finally, we attempt to put these components and pathways into signal transduction networks that are grouped into three generalized signaling types.

General Stress Signal Transduction Pathways

A generic signal transduction pathway starts with signal perception, followed by the generation of second messengers (e.g., inositol phosphates and reactive oxygen species [ROS]). Second messengers can modulate intracellular Ca^{2+} levels, often initiating a protein phosphorylation cascade that finally targets proteins directly involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes (Figure 1). The products of these genes may participate in the generation of regulatory molecules like the plant hormones abscisic acid (ABA), ethylene, and salicylic acid (SA). These regulatory molecules can, in turn, initiate a second round of signaling that may follow the above generic pathway, although different components are often involved (Figures 1 and 2).

Signal transduction requires the proper spatial and temporal coordination of all signaling molecules. Thus, there are certain molecules that participate in the modification, deliv-

ery, or assembly of signaling components, but do not directly relay the signal. They too are critical for the accurate transmission of stress signals. These proteins include protein modifiers (e.g., enzymes for protein lipidation, methylation, glycosylation, and ubiquitination), scaffolds, and adaptors (Xiong and Zhu, 2001) (Figure 1).

Multiplicity of Abiotic Stresses as Signals for Plants and the Need for Multiple Sensors

Low temperature, drought, and high salinity are very complex stimuli that possess many different yet related attributes, each of which may provide the plant cell with quite different information. For example, low temperature may immediately result in mechanical constraints, changes in activities of macromolecules, and reduced osmotic potential in the cellular milieu. High salinity includes both an ionic (chemical) and an osmotic (physical) component. The multiplicity of information embedded in abiotic stress signals underlies one aspect of the complexity of stress signaling.

On the basis of this multiplicity, it is unlikely that there is only one sensor that perceives the stress condition and controls all subsequent signaling. Rather, a single sensor might only regulate branches of the signaling cascade that are initiated by one aspect of the stress condition. For example, low temperature is known to change membrane fluidity (Murata and Los, 1997). A sensor detecting this change would initiate a signaling cascade responsive to membrane fluidity but would not necessarily control signaling initiated by an intracellular protein whose conformation/activity is directly altered by low temperature. Thus, there may be multiple primary sensors that perceive the initial stress signal.

Secondary signals (i.e., hormones and second messengers) can initiate another cascade of signaling events, which can differ from the primary signaling in time (i.e., lag behind) and in space (e.g., the signals may diffuse within or among cells, and their receptors may be in different subcellular locations from the primary sensors) (Figure 2). These secondary signals may also differ in specificity from primary stimuli, may be shared by different stress pathways, and may underlie the interaction among signaling pathways for different stresses and stress cross-protection. Therefore, one primary stress condition may activate multiple signaling pathways

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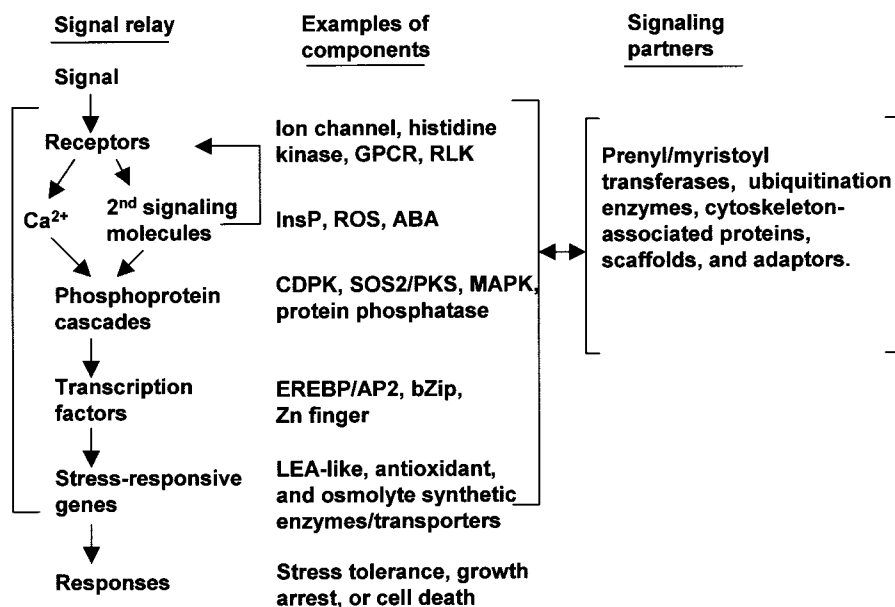


Figure 1. A Generic Pathway for the Transduction of Cold, Drought, and Salt Stress Signals in Plants.

Examples of signaling components in each of the steps are shown (for more detailed information, see Xiong and Zhu, 2001). Secondary signaling molecules can cause receptor-mediated Ca^{2+} release (indicated with a feedback arrow). Examples of signaling partners that modulate the main pathway are also shown. These partners can be regulated by the main pathway. Signaling can also bypass Ca^{2+} or secondary signaling molecules in early signaling steps. GPCR, G-protein coupled receptor; InsP, inositol polyphosphates; RLK, receptor-like kinase. Other abbreviations are given in the text.

differing in time, space, and outputs. These pathways may connect or interact with one another using shared components generating intertwined networks.

Potential Sensors for Abiotic Stress Signals

Given the multiplicity of stress signals, many different sensors are expected, although none have been confirmed for cold, drought, or salinity. All three stresses have been shown to induce transient Ca^{2+} influx into the cell cytoplasm (reviewed by Sanders et al., 1999; Knight, 2000). Therefore, channels responsible for this Ca^{2+} influx may represent one type of sensor for these stress signals. The activation of certain Ca^{2+} channels by cold may result from physical alterations in cellular structures. This phenomenon was demonstrated in studies showing that cold-induced Ca^{2+} influx in plants occurs only following a rapid temperature drop (Plieth et al., 1999), and that membrane fluidity and cytoskeletal reorganization are involved in early cold signaling (Órvar et al., 2000; Sangwan et al., 2001; Wang and Nick, 2001).

Another type of membrane protein sensor for low temperature perception could be a two-component histidine kinase. Evidence suggests that the cyanobacterium histidine kinase Hik33 (Suzuki et al., 2000) and the *Bacillus subtilis*

histidine kinase DesK (Aguilar et al., 2001) are thermosensors that regulate desaturase gene expression in response to temperature downshifts. In the genome of *Arabidopsis thaliana*, several putative two-component histidine kinases have been identified (Urao et al., 2000), although no evidence has been reported for any of these histidine kinases as thermosensors.

In plants, cold, drought, and salt stresses all stimulate the accumulation of compatible osmolytes and antioxidants (Hasegawa et al., 2000). In yeast and in animals, mitogen-activated protein kinase (MAPK) pathways are responsible for the production of compatible osmolytes and antioxidants. These MAPK pathways are activated by receptors/sensors such as protein tyrosine kinases, G-protein-coupled receptors, and two-component histidine kinases. Among these receptor-type proteins, histidine kinases have been unambiguously identified in plants. An *Arabidopsis* histidine kinase, AtHK1, can complement mutations in the yeast two-component histidine kinase sensor SLN1, and therefore may be involved in osmotic stress signal transduction in plants (Urao et al., 1999). Understanding the *in vivo* function of AtHK1 and other putative histidine kinases and their relationship to osmotic stress-activated MAPK pathways will certainly shed light on osmotic stress signal transduction.

Pathways leading to the activation of late embryogenesis-

abundant (*LEA*)-type genes including the dehydration-responsive element (DRE)/C-repeat (CRT) class of stress-responsive genes may be different from the pathways regulating osmolyte production. The activation of *LEA*-type genes may actually represent damage repair pathways (Zhu, 2001; Xiong and Zhu, 2002). Because the activity of phospholipase C in plants might be regulated by G-proteins, and phosphoinositols modulate the expression of these *LEA*-like genes under cold, drought, and salt stress (see below), G-protein-associated receptors may exist and function in the perception of a secondary signal derived from these stresses. In this regard, analysis of stress signaling in the Arabidopsis $G\alpha$ mutant *gpa1* (Ullah et al., 2001; Wang et al., 2001) would be of interest. G-protein-associated receptors might also serve as one kind of membrane-bound receptors for ABA.

Intracellular Secondary Signal Molecules

One early response to low temperature, drought, and salinity stress in plant cells is a transient increase in cytosolic Ca^{2+} , derived from either influx from the apoplastic space or release from internal stores (Knight, 2000; Sanders et al., 1999). Internal Ca^{2+} release is controlled by ligand-sensitive Ca^{2+} channels. These ligands are second messengers that have been described in animal cells including, for example, inositol polyphosphates, cyclic ADP ribose, and nicotinic acid adenine dinucleotide phosphate. These molecules have all been found to be able to induce Ca^{2+} release in plant cells and, in particular, guard cells (reviewed by Schroeder et al., 2001). An important feature of the role of Ca^{2+} as a signal is the presence of repetitive Ca^{2+} transients. These transients may be generated both by first-round second messengers and by signaling molecules such as ABA that may themselves be produced as a result of cascades of early Ca^{2+} signals (Figure 2). These rounds of Ca^{2+} signals may have quite different signaling consequences and, therefore, physiological meaning.

Phospholipids

As the selective barrier between living cells and their environments, the plasma membrane plays a key role in the perception and transmission of external information. Upon osmotic stress, changes in phospholipid composition are detected in plants as well as in other organisms (reviewed by Munnik et al., 1998). However, during exposure to stress, the major role of phospholipids, the backbone of cellular membranes, may be to serve as precursors for the generation of second-messenger molecules. Whereas the relevant cleaving enzymes are the phospholipases A_2 , C, and D, the most studied is the phosphoinositide-specific phospholipase C (PI-PLC). PI-PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) upon activation. PIP_2 itself is a signal and may be involved in several processes, such as the recruitment of signaling complexes to specific membrane locations and their assembly (Martin, 1998). Hydrolysis of PIP_2 in animal cells has been shown to desensitize a G-protein-stimulated K^+ current (Kobrinisky et al., 2000). Thus, PIP_2 could directly affect cellular ion homeostasis. During osmotic stress, plant cells may increase the production of PIP_2 by upregulating the expression of *PI5K* (Mikami et al., 1998), a gene that encodes a phosphatidylinositol 4-phosphate 5-kinase functioning in the production of PIP_2 . Consistent with this observation, osmotic stress was found to rapidly increase PIP_2 levels in cultured Arabidopsis cells (Pical et al., 1999; DeWald et al., 2001). Drought or salt stress also upregulates the mRNA levels for certain PI-PLC isoforms (Hirayama et al., 1995; Kopka et al., 1998). This increase in PI-PLC expression could contribute to increased cleavage of PIP_2 to produce two important molecules, diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). Diacylglycerol and IP_3 are second messengers that can activate protein kinase C and trigger Ca^{2+} release, respectively.

In plants, the role of exogenous IP_3 in releasing Ca^{2+} from cellular stores has been widely reported (Sanders et al., 1999; Schroeder et al., 2001). Transient increases in IP_3 were found in plants upon exposure to light, pathogen,

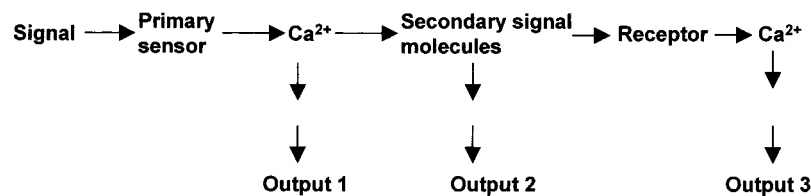


Figure 2. Repetitive Ca^{2+} Transients upon the Perception of a Primary Signal.

The primary increase in cytosolic Ca^{2+} facilitates the generation of secondary signaling molecules, which stimulate a second round of transient Ca^{2+} increases, both locally and globally. These second Ca^{2+} transients may feedback regulate each of the previous steps (not shown). Ca^{2+} transients from different sources may have different biological significance and result in different outputs, as shown. Secondary signaling molecules such as ROS can also directly regulate signal transduction without Ca^{2+} (Output 2).

gravity, anoxia, or several plant hormones (Munnik et al., 1998; Stevenson et al., 2000). IP_3 levels increase in Arabidopsis plants under salt stress, and the time frame for the increase correlates with changes in cytosolic Ca^{2+} levels (DeWald et al., 2001). Transient increases in IP_3 levels were also observed in plant tissues or cultured cells during salt stress (Srivastava et al., 1989; Drøbak and Watkins, 2000; Takahashi et al., 2001). Inhibition of PI-PLC activity eliminated transient IP_3 increases (DeWald et al., 2001; Takahashi et al., 2001) and inhibited the osmotic stress induction of the stress-responsive genes *RD29A* and *COR47* (Takahashi et al., 2001). The stress hormone ABA also elicits transient increases in IP_3 levels in *Vicia faba* guard cell protoplasts (Lee et al., 1996) and in Arabidopsis seedlings (Sanchez and Chua, 2001; Xiong et al., 2001c).

Given the critical role of IP_3 in signaling, cellular IP_3 levels must be tightly regulated through both controlled production and degradation. Biochemical studies suggest that in animal cells, IP_3 is degraded through either an inositol polyphosphate 3-kinase pathway or an inositol polyphosphate 5-phosphatase (Ins5Pase) pathway, resulting in the generation of inositol 1,3,4,5-tetraphosphate and inositol 1,4-bisphosphate [$Ins(1,4)P_2$], respectively (Majerus, 1992). However, information regarding the turnover of IP_3 in plants is limited. To study the relationship between IP_3 levels and gene expression, Burnette et al. (2001) overexpressed an Ins5Pase and found a delay in ABA induction of expression of a cold-induced gene (*KIN1*) in the transgenic plants. In an independent study, Sanchez and Chua (2001) overexpressed the Ins5Pase *AtP5P11* under control of an inducible promoter. They found that expression of *AtP5P11* reduced IP_3 accumulation in response to ABA treatment and decreased the induction of the expression of ABA-responsive genes such as *RD29A*, *KIN2*, and *RD22*. These results suggest that ABA-induced IP_3 generation contributes to the induction of these genes. Taken together, these studies indicate that modifying Ins5Pase dosage can regulate stimulus-induced endogenous IP_3 levels and affect stress and ABA signal transduction. In the Arabidopsis genome, there are ~15 putative Ins5Pases (compared to only 5 FRY1-like inositol polyphosphate 1-phosphatases [Ins1Pases], see below). It is likely that different isoforms might have different substrate specificities and/or subcellular localizations that imply distinct functions in the degradation of IP_3 generated in response to various stimuli. Clearly, the role of Ins5Pases in regulating inositol phosphate levels should be addressed with loss-of-function *ins5pase* mutants.

In a genetic screen using a firefly luciferase reporter under the control of the stress-responsive *RD29A* promoter (Ishitani et al., 1997; see below), Xiong et al. (2001c) isolated an Arabidopsis mutant *fiery1* (*fry1*) that exhibited an enhanced induction of stress-responsive genes under cold, drought, salt, and ABA treatments. Positional cloning of the *FRY1* gene revealed that it encodes a bifunctional enzyme with both 3'(2'),5'-bisphosphate nucleotidase and Ins1Pase activities. *FRY1* is identical to the previously described *SAL1*

gene that was isolated by its ability to confer increased salt tolerance when expressed in yeast cells (Quintero et al., 1996). Because *fry1* mutant plants did not show sulfur deficiency symptoms, the 3'(2'),5'-bisphosphate nucleotidase activity of *FRY1* that functions in sulfur assimilation appears dispensable. Therefore, it was hypothesized that changes in the Ins1Pase activity were responsible for the enhanced gene expression in *fry1* mutants in response to stress and ABA treatment (Xiong et al., 2001c). Results from these studies bring up interesting questions as to whether IP_3 in plants is degraded via a 5-phosphatase or a 1-phosphatase pathway, or both (Figure 3), and what the contribution of each pathway to the overall termination of IP_3 signaling might be.

Several studies have reported that inositol 4,5-bisphosphate is the primary and immediate catabolite of 3H -labeled IP_3 in plants (Joseph et al., 1989; Drøbak et al., 1991; Brearley et al., 1997), suggesting that in these plants, IP_3 was first hydrolyzed through a 1-phosphatase pathway. However, the Ins1Pase responsible for this early termination of the IP_3 signal in plants has not been identified. In addition, the Ins1Pases characterized in most animal cells do not hydrolyze IP_3 (Inhorn et al., 1987; Majerus, 1992). In the cell types in animals where the 1-phosphatases might be the primary terminators of IP_3 signals (e.g., Lynch et al., 1997), the molecular identities of these phosphatases are still unknown. In Arabidopsis, the activity of *FRY1/SAL1* in the hydrolysis of $Ins(1,4)P_2$ and inositol 1,3,4-trisphosphate [$Ins(1,3,4)P_3$] was demonstrated previously (Quintero et al., 1996), but whether it could hydrolyze IP_3 was not known. Using IP_3 as a substrate, *FRY1* recombinant protein was found to have a measurable albeit limited activity [$\sim 13\%$ relative to its ability to hydrolyze $Ins(1,4)P_2$ or $Ins(1,3,4)P_3$] (Xiong et al., 2001c). The in vivo activity of *FRY1* on IP_3 and its significance in overall IP_3 metabolism have yet to be determined. Nevertheless, even without an activity on IP_3 directly, loss of *FRY1* would inevitably slow down IP_3 degradation by blocking further degradation of $Ins(1,4)P_2$ and $Ins(1,3,4)P_3$ (Figure 3). Measurement of IP_3 levels in *fry1* and wild-type plants treated with ABA indicated that, whereas ABA induced a transient increase in IP_3 levels in wild-type plants, the IP_3 levels in *fry1* mutant plants were higher and more sustained (Xiong et al., 2001c). Sustained IP_3 levels likely contributed to the enhanced expression of stress-responsive genes in *fry1* mutant plants. It is interesting to note that whereas the expression of genes including *RD29A*, *KIN1*, *COR15A*, *HSP70* and *ADH* was enhanced in the *fry1* mutant, the induction of another stress-responsive gene, *COR47*, was not enhanced compared with its expression in the wild type. This implies that *COR47* might be regulated through a pathway different from that used by the other genes (Xiong et al., 2001c).

Accumulating evidence suggests that phospholipase D (PLD) is also involved in the transduction of stress signals. PLD hydrolyzes phospholipids to generate phosphatidic acid (PA), another second messenger in animal cells that can activate PI-PLC and protein kinase C (English, 1996). PA

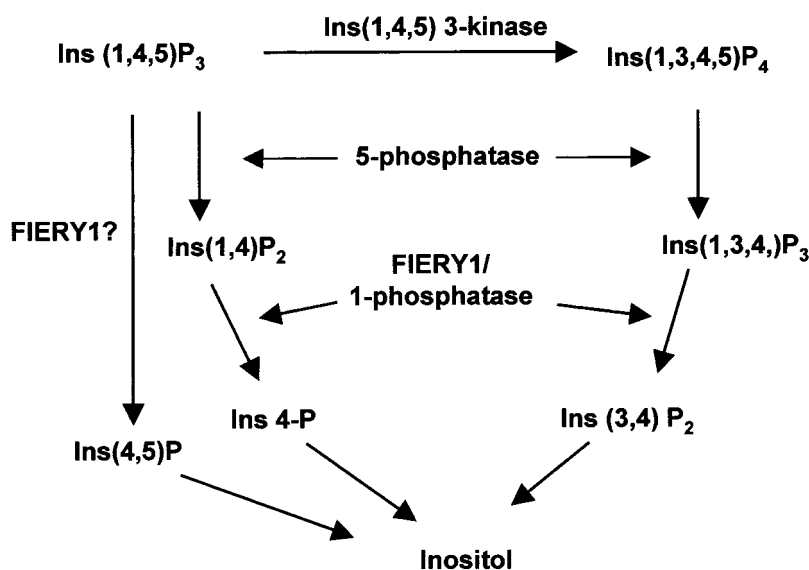


Figure 3. Potential Pathways for Inositol 1,4,5-Trisphosphate (IP_3) Degradation in Plants.

The pathways are drawn on the basis of information from animal systems. FIERY1 inositol polyphosphate 1-phosphatase can hydrolyze $Ins(1,4)P_2$ and $Ins(1,3,4)P_3$. A potential pathway mediated by FIERY1 with direct hydrolysis of IP_3 at the 1-position is also indicated (with a question mark). 5-phosphatase, inositol polyphosphate 5-phosphatase.

may also serve as a messenger in plants (Wang, 1999). In guard cell protoplasts, PLD activity mediates ABA-induced stomatal closure (Jacob et al., 1999). Drought and hyperosmolarity activate PLD and lead to transient increases in PA levels in plants (Frank et al., 2000; Munnik et al., 2000; Katagiri et al., 2001). PLD appears to be activated by osmotic stress through a G-protein (Frank et al., 2000) independently of ABA (Frank et al., 2000; Katagiri et al., 2001). However, excess PLD activity may have a negative impact on plant stress tolerance. PA is a nonbilayer lipid favoring hexagonal phase formation and may destabilize membranes at high concentrations (Wang, 1999). Drought stress-induced PLD activities were found to be higher in drought-sensitive than in drought-tolerant cultivars of cowpea (El Maarouf et al., 1999), suggesting that a high PLD activity may jeopardize membrane integrity. Consistent with this notion, *Arabidopsis* deficient in $PLD\alpha$ was found to be more tolerant to freezing stress (X. Wang, personal communication).

ROS

Drought, salt, and cold stress all induce the accumulation of ROS such as superoxide, hydrogen peroxide, and hydroxyl radicals (e.g., Hasegawa et al., 2000). These ROS may be signals inducing ROS scavengers and other protective mechanisms, as well as damaging agents contributing to stress injury in plants (e.g., Prasad et al., 1994). Because

ABA was shown to induce H_2O_2 production (Guan et al., 2000; Pei et al., 2000), ROS may be intermediate signals for ABA in mediating Catalase 1 gene (*CAT1*) expression (Guan et al., 2000), thermotolerance (Gong et al., 1998), activation of Ca^{2+} channels in guard cells (Pei et al., 2000), stomatal closure (e.g., Pei et al., 2000; Zhang et al., 2001), and even ABA biosynthesis (Zhao et al., 2001). While it is possible that ROS may activate downstream signal cascades via Ca^{2+} (e.g., Price et al., 1994), it is also possible that they can be sensed directly by key signaling proteins such as a tyrosine phosphatase through oxidation of conserved cysteine residues (reviewed by Xiong and Zhu, 2002). In animal cells, reduced tyrosine phosphatase activity causes an increase in the output of MAPK pathways because tyrosine phosphatases inhibit MAPKs through dephosphorylation (Rhee et al., 2000).

It is clear that ROS contribute to stress damage, as evidenced by observations that transgenic plants overexpressing ROS scavengers or mutants with higher ROS scavenging ability show increased tolerance to environmental stresses (reviewed by Bohnert and Sheveleva, 1998; Nuccio et al., 1999; Hasegawa et al., 2000; Kocsy et al., 2001). Whereas the connections between ROS signal transduction and osmotic stress signal transduction are just beginning to emerge (Xiong and Zhu, 2002), the involvement of ROS in pathogenesis signal transduction is well-documented (Lamb and Dixon, 1997). In hypersensitive responses, SA is thought to potentiate ROS signaling (Klessig et al., 2000). Although it

is unclear whether osmotic stress leads to an increased SA level in plants, the observation that osmotic stress and SA activate the same MAPK (Hoyos and Zhang, 2000; Mikolajczyk et al., 2000; see below) suggests that the osmotic stress signal transduction and SA signal transduction may employ certain common components. Using transgenic Arabidopsis expressing a salicylate hydroxylase (*NahG*) gene, Borsani et al. (2001) demonstrated that these SA-deficient seedlings are more tolerant to salt and other osmotic stress. They suggested that the increased osmotic stress tolerance might result from decreased SA-mediated ROS generation in the *NahG*-expressing plants.

Some genes related to osmotic stress signaling have been shown to be upregulated by oxidative stress, including the transcription factor *DREB2A* (see below) and a histidine kinase (Desikan et al., 2001). It is not known whether other histidine kinases, such as AtHK1, that are potentially involved in osmotic stress signal transduction (Urao et al., 1999) are regulated by oxidative stress. Evidence from animal and yeast studies suggests that the histidine kinase-activated osmosensing MAPK pathways also mediate ROS signaling (reviewed by Xiong and Zhu, 2002). In Arabidopsis culture cells, it was reported that the MAPK AtMPK6 that can be activated by low temperature and osmotic stress could also be activated by oxidative stress (Yuasa et al., 2001). Thus, it is likely that potential MAPK modules that mediate osmotic stress signal transduction may also be used for ROS signaling in plants. On the other hand, the significance of oxidative stress-regulated *DREB2A* expression in osmotic stress responses is unclear. Oxidative stress does not seem to activate genes of the DREB2A-targeted DRE/CRT class, such as *RD29A* (J.K. Zhu, unpublished data). Additionally, Arabidopsis plants overexpressing the MAP kinase kinase kinase (MAPKKK) *ANP1* were not affected in the expression of *RD29A*, although these plants had a higher ROS scavenging capacity and an increased salt tolerance (Kovtun et al., 2000). Thus, MAPK pathways and the pathways for the activation of *LEA*-like genes may represent different signaling types.

Ca²⁺-Coupled Phosphoprotein Cascades

Transient increases in cytosolic Ca²⁺ are perceived by various Ca²⁺ binding proteins. In the case of abiotic stress signaling, evidence suggests that Ca²⁺-dependent protein kinases (CDPKs) and the SOS3 family of Ca²⁺ sensors are major players in coupling this universal inorganic signal to specific protein phosphorylation cascades. CDPKs are serine/threonine protein kinases with a C-terminal calmodulin-like domain with up to 4 EF-hand motifs that can directly bind Ca²⁺. Some CDPKs have an N-terminal myristoylation motif suggesting potential association with membranes. Indeed, CDPKs from rice (*OsCpPK2*) and zucchini (*CpCpK1*) were shown to be myristoylated and palmitoylated and targeted to membrane fractions (Ellard-Ivey et al., 1999; Martin

and Busconi, 2000). The Arabidopsis genome encodes at least 34 putative CDPKs (Harmon et al., 2001). A number of studies have shown that CDPKs are induced or activated by abiotic stresses, suggesting that they may be involved in abiotic stress signaling (Urao et al., 1994; Pei et al., 1996; Tähtiharju et al., 1997; Hwang et al., 2000). In rice plants, a membrane-associated CDPK was activated by cold treatment (Martin and Busconi, 2001). In addition, overexpression of *OsCpPK7* resulted in increased cold and osmotic stress tolerance in rice (Saijo et al., 2000). Thus, CDPKs somehow play roles in the development of stress tolerance. A clear demonstration of the involvement of CDPK in stress signal transduction has come from experiments in which an active AtCDPK1 induced the expression of the stress-responsive *HVA1* promoter-driven reporter gene in maize leaf protoplasts (Sheen, 1996). Interestingly, a protein phosphatase type 2C (*AtPP2CA*) can block AtCDPK1 activation of the *HVA*-driven reporter gene expression (Sheen, 1996, 1998). It is unclear whether *AtPP2CA* acts directly on AtCDPK1 or modulates a downstream phosphorylation cascade. Recently, Tähtiharju and Palva (2001) generated *AtPP2CA*-silenced Arabidopsis plants and found that there was an enhanced induction of *CBF1*, *RAB18*, *RCI2A*, and *LT178* (i.e., *RD29A*) gene expression in the silenced lines under cold or ABA treatment, and the transgenic plants exhibited a higher degree of cold acclimation.

Regarding the role of CDPK in stress signal transduction, there is ambiguity about how it might connect with other signaling modules. A CDPK was activated in response to pathogen infection (Romeis et al., 2000), yet its relationship to MAPK pathways that are also activated during the resistance responses is unclear. Results from previous studies in animals and yeast also lack a clear connection between Ca²⁺ binding protein/calmodulin and MAPK pathways. Recent studies with neural cells suggest that calmodulin perceives local Ca²⁺ and activates a MAPK pathway to regulate target gene expression (Dolmetsch et al., 2001), although the connecting point between Ca²⁺-calmodulin and the MAPK pathway remains unknown. In plants, an interesting finding was reported by Patharkar and Cushman (2000). These researchers obtained a CDPK-interacting protein (CSP1) from a yeast two-hybrid screen. CSP1 is a two-component pseudo-response regulator protein that could serve as a transcriptional activator (see below), suggesting a potential role for CDPK in directly shuttling information to the nucleus to activate gene expression.

An important group of Ca²⁺ sensors in plants is the SOS3 family of Ca²⁺ binding proteins. The amino acid sequence of SOS3 is most closely related to the regulatory subunit of yeast calcineurin (CNB) and animal neuronal calcium sensors (Liu and Zhu, 1998). A loss-of-function mutation in the Arabidopsis SOS3 gene renders the mutant plants hypersensitive to NaCl. Interestingly, the salt-hypersensitive phenotype of *sos3* mutant plants can be partially rescued by increased concentrations of Ca²⁺ in growth media (Liu and Zhu, 1997a). Thus, SOS3 may underlie part

of the molecular basis for the long-observed phenomenon that higher external Ca^{2+} can alleviate salt toxicity in plants (Zhu, 2000).

SOS3 possesses three EF-hand motifs and binds Ca^{2+} with low affinity compared with caltractin or calmodulin (Ishitani et al., 2000). The *sos3* mutation occurs in one of the EF-hand motifs and thus impairs the ability of the protein to bind Ca^{2+} (Liu and Zhu, 1998; Ishitani et al., 2000). The low Ca^{2+} binding affinity of SOS3 suggests that the function of SOS3 in salt tolerance may be realized at specific subcellular locations in which transient increases in Ca^{2+} are very large. SOS3 is myristoylated *in vivo*, and myristoylation is required for its function in salt tolerance, because disruption of the myristoylation motif eliminated the ability of SOS3 to complement the salt-sensitive phenotype of *sos3* mutant plants (Ishitani et al., 2000). The requirement for myristoylation suggests that SOS3 may regulate the activities of membrane-bound ion transporters. This is supported by the identification of additional salt tolerance loci SOS2 and SOS1 in Arabidopsis, as discussed below.

Arabidopsis *sos2* and *sos1* mutants, like *sos3*, are hypersensitive to salt stress and were isolated by their retarded growth on NaCl-supplemented agar plates (Wu et al., 1996; Zhu et al., 1998). SOS2 is a serine/threonine protein kinase with an SNF1/AMPK-like catalytic domain and a unique regulatory domain (Liu et al., 2000). The catalytic and regulatory domains of SOS2 interact with one another and repress the kinase activity, presumably by blocking substrate access to the catalytic site (Guo et al., 2001). Interestingly, SOS3 interacts with SOS2 through the regulatory domain of SOS2, and this may relieve the repression of kinase activity by making the catalytic site accessible to substrates (Halfter et al., 2000; Guo et al., 2001). Deletion analysis identified a 21-amino-acid sequence (FISL motif) in the regulatory domain as necessary and sufficient for interaction with SOS3 (Guo et al., 2001). Deletion of the regulatory domain (Guo et al., 2001) or the FISL motif results in a constitutively active kinase. An activated form of SOS2 can also be generated by replacing Thr-168 in the putative activation loop with Asp (Guo et al., 2001). When introduced into plants under control of the cauliflower mosaic virus 35S promoter, this active form of SOS2 can complement the salt-hypersensitive phenotypes of *sos2* and *sos3* (Y. Guo and J.-K. Zhu, unpublished results).

Studies comparing the growth of wild-type and mutant plants in response to NaCl, and sequence analysis of the predicted SOS1 protein suggested that SOS1 encodes a Na^+/H^+ exchanger (antiporter) on the plasma membrane (Shi et al., 2000). Genetic analysis indicated that SOS1, SOS2 and SOS3 function in a common pathway in controlling salt tolerance (Zhu et al., 1998; Halfter et al., 2000), and functional studies in yeast and plants have shown that SOS1 is activated by the SOS3–SOS2 complex. When SOS1 alone was introduced into a yeast mutant lacking all endogenous Na^+ -ATPases and Na^+/H^+ exchangers, the salt tolerance of the yeast mutant was only enhanced slightly

(Shi et al., 2002). However, when SOS1 was coexpressed with SOS2 and SOS3, or activated SOS2 was introduced, the yeast transformants became substantially more tolerant to salt (J. Pardo and J.-K. Zhu, unpublished data). Plasma membrane vesicles isolated from *sos* mutant plants had very low Na^+/H^+ exchange activity compared with the activity in vesicles isolated from wild-type plants. When activated SOS2 protein was added to membrane vesicles isolated from mutant plants, exchange activity was unaffected in the *sos1* mutant but increased to near wild-type levels in the *sos2* and *sos3* mutants (Qiu et al., 2002). These results demonstrate that upon activation by SOS3, SOS2 stimulates the Na^+/H^+ exchange activity of SOS1.

In addition to regulating SOS1 exchange activity, SOS3–SOS2 may regulate other salt tolerance effectors. One such effector might be the Na^+ transporter AtHKT1 (Uozumi et al., 2000). HKT1 homologs in other plant species were suggested to be either K^+ transporters or Na^+/K^+ cotransporters (Rubio et al., 1995; Horie et al., 2001; Liu et al., 2001). In Arabidopsis, mutations in AtHKT1 suppressed the salt hypersensitivity phenotype of *sos3* (Rus et al., 2001), suggesting that wild-type SOS3 may inhibit the activity of AtHKT1 as a Na^+ influx transporter. Several other salt stress-related genes whose expression is uniquely regulated by SOS3–SOS2 have been identified (Gong et al., 2001). Genome-wide expression profiling of *sos2* and *sos3* mutants should identify more genes that are regulated at the transcriptional level by the SOS pathway.

Because the SOS pathway operates during ionic stress, it is thought that homologs of SOS3 and SOS2 may also function in the transduction of other stress or hormonal signals. Including SOS2 and SOS3, Arabidopsis has eight SOS3-like Ca^{2+} binding proteins and 22 SOS2-like protein kinases (Guo et al., 2001), some of which have been found to interact in yeast two-hybrid assays (Albrecht et al., 2001; Guo et al., 2001).

Other Phosphoprotein Signaling Pathways

In addition to Ca^{2+} -regulated protein kinase pathways, plants also use other phosphoprotein modules for abiotic stress signaling. In yeast, the HOG1 MAPK pathway is activated in response to hyperosmolarity and is responsible for increased production of osmolytes such as glycerol that are important for osmotic adjustment. It is possible that similar pathways also exist in plants, as indicated by osmotic stress activation of some MAPK pathway components, although the plant pathway outputs are unclear at this time.

Parts of several MAPK modules (i.e., MAPKKK–MAPKK–MAPK) that may be involved in osmotic stress signaling have been identified in alfalfa (SIMKK–SIMK; Kiegerl et al., 2000) and in tobacco (NtMEK2–SIPK/WIPK; Yang et al., 2001) (Zhang and Klessig, 2001). Except for activation by stress treatment, however, the *in planta* function during

stress signaling has not been established for any of the potential MAPK pathways. Salt stress can activate different MAPKs at different times after the onset of stress, and the activities of these MAPKs also last for different time periods (e.g., Mikolajczyk et al., 2000). Additionally, different levels of salt stress can cause the activation of distinct MAPKs (Munnik et al., 1999). In Arabidopsis, one proposed MAPK pathway involved in stress signal transduction is AtMEKK1-MEK1/AtMCK2-AtMPK4 (Ichimura et al., 2000). AtMPK4 is rapidly activated by cold, hyposmolarity, or wounding. Petersen et al. (2000) isolated an Arabidopsis *atmpk4* mutant that showed constitutive systemic acquired resistance to pathogens. The *mpk4* mutant could be an invaluable genetic tool for testing the role of this MAPK in osmotic stress signal transduction, and for identifying the pathway targets. However, because this mutant has a high constitutive SA concentration (Petersen et al., 2000), analysis of osmotic stress signaling in the mutant may be complicated by the fact that SA potentiates osmotic stress damage (Borsani et al., 2001). In any case, analysis of the *in vivo* function of the various MAPK components and their interrelationships will be essential for constructing signaling pathways. Recently, an Arabidopsis mutant defective in a MAPK phosphatase and that exhibited hypersensitivity to ultraviolet C was identified (Ulm et al., 2001). The sensitivity of this mutant to salt stress was reported to be unaffected relative to wild-type plants (Ulm et al., 2001). Thus, mutations in any MAPK module that affect osmotic stress signaling remain to be described.

A common observation both in plants and in other organisms is that one MAPK module can be used for the transmission of multiple signals. For example, the SA-induced protein kinase is activated by SA and wounding (Zhang and Klessig, 1998) as well as by osmotic stress (Mikolajczyk et al., 2000). Oxidative stress also activates the MAPKKK NPK1 (or the Arabidopsis ANP1) that targets two MAPKs, AtMPK3 and AtMPK6. Overexpression of NPK1 in Arabidopsis plants resulted in the activation of oxidative stress-responsive genes and increased tolerance of the transgenic plants to freezing, salt, and heat stress (Kovtun et al., 2000), probably due to increased oxidative scavenging ability.

In addition to MAPK pathways, other protein kinases are also involved in osmotic stress signal transduction. For example, a tobacco Arabidopsis serine/threonine kinase 1 (ASK1)-like protein kinase was activated within 1 min after osmotic stress (Mikolajczyk et al., 2000). ASK1 has sequence similarity to the soybean protein kinases SPK1 and SPK2. Interestingly, the soybean SPK1 and SPK2 were found to be activated by osmotic stress and able to phosphorylate a phosphatidylinositol transfer protein, Ssh1p (Monks et al., 2001). Ssh1p was rapidly phosphorylated upon osmotic stress and it, in turn, enhanced phosphatidylinositol 3-kinase and 4-kinase activities (Monks et al., 2001). SPK1 and SPK2 may thus modulate osmotic stress signaling through regulation of phosphoinositide metabolism.

ABA and Stress Signal Transduction Networks

During biotic or abiotic stress, plants produce increased amounts of hormones such as ABA and ethylene. In addition, SA and perhaps jasmonic acid may be involved in some parts of stress responses. These hormones may interact with one another in regulating stress signaling and plant stress tolerance. For example, ethylene has been shown to enhance ABA action in seeds (Gazzarrini and McCourt, 2001) but may counteract ABA effects in vegetative tissues under drought stress (Spollen et al., 2000). Nonetheless, ABA is undoubtedly the plant hormone most intimately involved in stress signal transduction.

A Stress- and ABA-Signaling Network Revealed by Genetic Analysis

The involvement of ABA in plant environmental stress responses has long been recognized. However, the extent and the molecular basis of ABA involvement in stress-responsive gene expression and stress tolerance were not immediately clear. Studies of the relationship between ABA and different stress-signaling pathways have been hampered by the paucity of signaling mutants. To facilitate genetic screens for stress-signaling mutants, transgenic Arabidopsis were engineered that express the firefly luciferase reporter gene (*LUC*) under control of the *RD29A* promoter, which contains both ABA- (ABA-responsive element [ABRE]) and dehydration-responsive elements (DRE/CRT). Seed from the *RD29A-LUC* transgenic plants were mutagenized with ethyl methane-sulfonate or T-DNA, and seedlings from mutagenized populations were screened for altered *RD29A-LUC* responses (luminescence intensity) in response to stress and ABA treatments (Ishitani et al., 1997). Compared with wild-type *RD29A-LUC* plants, mutants exhibited either a constitutive (*cos*), high (*hos*), or low (*los*) level of *RD29A-LUC* expression in response to various stress or ABA treatments. The occurrence of mutations with differential responses to stress or ABA or combinations of the stimuli revealed a complex signal transduction network and suggest that there are extensive connections among cold, drought, salinity, and ABA signal transduction pathways (Ishitani et al., 1997). The characterization and cloning of some of the mutations have begun to provide new insights into the mechanisms of stress and ABA signal transduction.

Dependence of Stress Signaling on ABA

Salt, drought, and to some extent, cold stress cause an increased biosynthesis and accumulation of ABA, which can be rapidly catabolized following the relief of stress (Koorneef et al., 1998; Cutler and Krochko, 1999; Liotenberg et al., 1999; Taylor et al., 2000). Many stress-responsive genes are

upregulated by ABA (Ingram and Bartel, 1996; Bray, 1997; Rock, 2000). The role of ABA in osmotic stress signal transduction was previously addressed by studying the stress induction of several of these genes in the Arabidopsis ABA-deficient mutant *aba1-1* and dominant ABA-insensitive mutants *abi1-1* and *abi2-1*. A general conclusion from these studies was that whereas low-temperature-regulated gene expression is relatively independent of ABA, osmotic stress-regulated genes can be activated through both ABA-dependent and ABA-independent pathways (Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). However, recent genetic evidence suggests that stress-signaling pathways for the activation of *LEA*-like genes that are completely independent of ABA may not exist. In genetic screens, a group of mutants that exhibit diminished expression of *RD29A-LUC* under osmotic stress compared with wild-type plants was recovered (Ishitani et al., 1997). Two of the loci defined by these mutants, *LOS5* and *LOS6*, have been characterized and the genes isolated. In *los5*, the expression of several stress-responsive genes, such as *RD29A*, *COR15*, *COR47*, *RD22*, and *P5CS*, was severely reduced or even completely blocked during salt stress (Xiong et al., 2001b). Interestingly, *los5* plants are defective in drought-induced ABA biosynthesis. Molecular cloning revealed that *LOS5* encodes a molybdenum cofactor sulfurase (MCSU) and is allelic to *ABA3* (Xiong et al., 2001b). The *ABA3* locus was defined previously by the *aba3-1* and *aba3-2* mutants (Léon-Kloosterziel et al., 1996) and recently by another allele *frs1-1* (Llorente et al., 2000). When exogenous ABA was applied, salt induction of *RD29A-LUC* was restored to the wild-type level, demonstrating that ABA deficiency was responsible for the defect in osmotic stress regulation of gene expression (Xiong et al., 2001b). These findings suggest that osmotic stress induction of these stress-responsive genes is almost entirely dependent on ABA.

Similarly, in *los6* mutant plants, osmotic stress induction of *RD29A*, *COR15A*, *KIN1*, *COR47*, *RD19*, and *ADH* was lower than that in wild-type plants (Xiong et al., 2002). *los6* plants are also defective in drought-induced ABA biosynthesis. Genetic analysis showed that *los6* is allelic to *aba1* and codes for a zeaxanthin epoxidase (ZEP) (Xiong et al., 2002; see below).

Characterizations of the *los5* and *los6* mutants have revealed a critical role for ABA in mediating osmotic stress regulation of gene expression. Because ABA deficiency does not appear to significantly affect the expression of *DREB2A* (which codes for a drought stress-specific transcription factor; see below), it is thought that ABA signaling may be required for regulating the activity of *DREB2A* or its associated factors in the activation of the DRE class of genes (Xiong et al., 2001b) (Figure 4). This level of interaction between ABA signaling and osmotic stress signaling may underlie the synergistic interaction between ABA and osmotic stress in activating the expression of stress-responsive genes (Bostock and Quatrano, 1992; Xiong et al., 1999a, 1999b, 2001b).

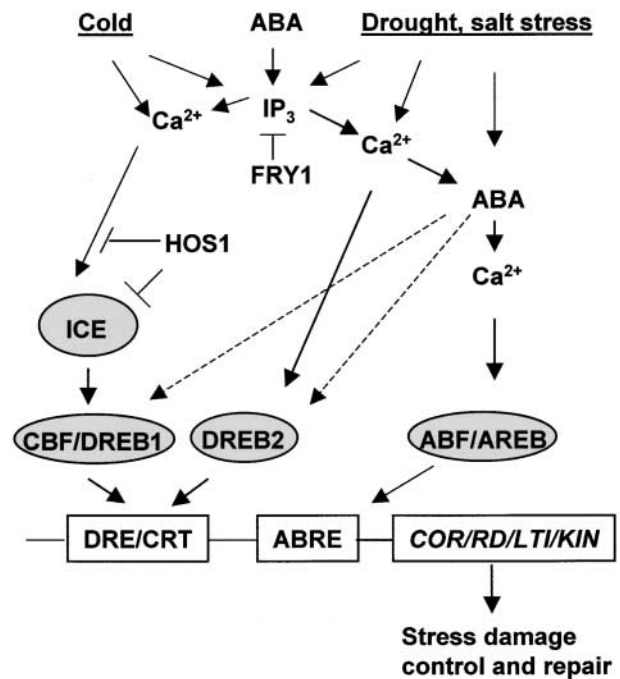


Figure 4. Pathways for the Activation of the *LEA*-Like Class of Stress-Responsive Genes with DRE/CRT and ABRE *cis* Elements.

Cold, drought, salt stress, and ABA can activate these genes through stress-inducible transcription factors CBF/DREB1 and DREB2, and ABA-inducible bZIP transcription factors ABF/AEB (Shinozaki and Yamaguchi-Shinozaki, 2000). An unidentified transcriptional activator, ICE (inducer of CBF expression) (Thomashow, 2001), is indicated. IP₃ is involved in the signaling, as revealed by genetic identification of the *FRY1* locus, which negatively regulates IP₃ levels and stress signaling (Xiong et al., 2001c). The *HOS1* locus negatively regulates cold signaling, presumably by targeting ICE or upstream signaling components for degradation (Lee et al., 2001). DREB2-mediated gene activation also depends on ABA-dependent posttranscriptional/translational modifications of CBF/DREB1 or DREB2 or associated coactivators (indicated with dashed arrows). *COR*, cold regulated; *KIN*, cold induced; *LTI*, low-temperature induced; *RD*, responsive to dehydration.

Regulation of ABA Biosynthetic Genes

Increased ABA levels under drought and salt stress are mainly achieved by the induction of genes coding for enzymes that catalyze ABA biosynthetic reactions. The ABA biosynthetic pathway in higher plants is understood to a great extent (reviewed by Koornneef et al., 1998; Lietenberg et al., 1999; Taylor et al., 2000; Milborrow, 2001) (Figure 5). ZEP (encoded by *ABA1* in Arabidopsis and *ABA2* in tobacco; Marin et al., 1996) catalyzes the epoxidation of zeaxanthin and antheraxanthin to violaxanthin (Duckham et al., 1991; Rock and Zeevaart, 1991). The 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyzes the oxidative cleavage of

9-*cis*-neoxanthin to generate xanthoxin (Schwartz et al., 1997b; Tan et al., 1997). It is thought that xanthoxin is converted to ABA by a two-step reaction via ABA-aldehyde. The *Arabidopsis aba2* mutant is impaired in the first step of this reaction, and is thus unable to convert xanthoxin into ABA-aldehyde (Léon-Kloosterziel et al., 1996). The *Arabidopsis aba3* mutant is defective in the last step of ABA biosynthesis, i.e., the conversion of ABA-aldehyde to ABA (Schwartz et al., 1997a; Bittner et al., 2001), which is catalyzed by ABA-aldehyde oxidase (AAO) (Figure 5). Mutations in either the aldehyde oxidase apoprotein (e.g., Seo et al., 2000) or molybdenum cofactor biosynthetic enzymes would impair ABA biosynthesis and lead to ABA deficiency in plants. In this ABA biosynthetic pathway, the rate-limiting step was thought to be the oxidative cleavage of neoxanthin catalyzed by NCED (Tan et al., 1997; Liotenberg et al., 1999; Qin and Zeevaart, 1999; Taylor et al., 2000; Thompson et al., 2000).

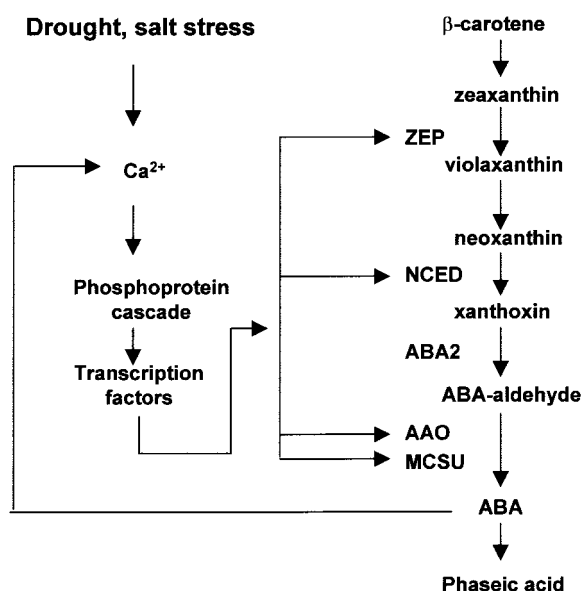


Figure 5. Pathway and Regulation of ABA Biosynthesis.

ABA is synthesized from a C_{40} precursor β -carotene via the oxidative cleavage of neoxanthin and a two-step conversion of xanthoxin to ABA via ABA-aldehyde. Environmental stress such as drought, salt and, to a lesser extent, cold stimulates the biosynthesis and accumulation of ABA by activating genes coding for ABA biosynthetic enzymes. Stress activation of ABA biosynthetic genes is probably mediated by a Ca^{2+} -dependent phosphorelay cascade, as shown at left. In addition, ABA can feedback stimulate the expression of ABA biosynthetic genes, also likely through a Ca^{2+} -dependent phosphoprotein cascade (Xiong et al., 2001a, 2002; L. Xiong and J.K. Zhu, unpublished data). Also indicated is the breakdown of ABA to phaseic acid. AAO, ABA-aldehyde oxidase; MCSU, molybdenum cofactor sulfurylase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; ZEP, zeaxanthin epoxidase.

Expression studies with *ZEP*, *NCED*, *AAO3*, and *MCSU* indicated that these genes are all upregulated by drought and salt stress (Audran et al., 1998; Seo et al., 2000; Iuchi et al., 2001; Xiong et al., 2001b, 2002), although their protein levels were not examined in every case. The expression of *ZEP* (Xiong et al., 2002), *NCED* (Qin and Zeevaart, 1999), and *MCSU* (Xiong et al., 2001b) was not obviously upregulated by cold, consistent with little or no increase in ABA content in plants subjected to cold treatment.

ABA has long been thought to be able to activate enzymes that function in ABA catabolism. Indeed, the activity of a cytochrome P450 enzyme ABA 8'-hydroxylase, which catalyzes the first step of ABA degradation, was stimulated by exogenous ABA (e.g., Krochko et al., 1998). However, whether and how ABA regulates its own biosynthetic genes is not clear. Interestingly, except for NCEDs, whose expression is not significantly induced by ABA treatment (Iuchi et al., 2001; Xiong et al., 2001a), *ZEP* (i.e., *LOS6/ABA1*), *AAO3*, and *MCSU* (i.e., *LOS5/ABA3*), genes are all upregulated by ABA (Xiong et al., 2001a, 2001b, 2002). This suggests that positive feedback regulation of ABA biosynthesis by ABA exists, underscoring a novel stress adaptation mechanism in which an initial induction of ABA biosynthesis may rapidly stimulate further biosynthesis of ABA through a positive feedback loop (Figure 5). This feedback loop is indirectly regulated by *SAD1* (supersensitive to ABA and drought 1), since the *sad1* mutation impairs ABA regulation of *AAO3* and *MCSU* genes (Xiong et al., 2001a). In addition, in the ABA-insensitive mutant *abi1*, this feedback loop is partially impaired, but it is unaffected in *abi2* (Xiong et al., 2002). The observation that ROS may mediate both ABA signaling (see above) and ABA biosynthesis (Zhao et al., 2001) suggests that the feedback regulation of ABA biosynthetic genes by ABA may be mediated in part by ROS through a protein phosphorylation cascade (Figure 5). The significance of this feedback regulation in ABA biosynthesis under abiotic stress awaits future study. Assuming that this feedback loop is important in regulating overall ABA biosynthesis, the fact that NCEDs are either not upregulated or weakly upregulated by ABA is consistent with the notion that NCED catalyzes a limiting step in ABA biosynthesis. Nonetheless, the observation that overexpression of either one of these ABA biosynthetic genes led to increased ABA biosynthesis and enhanced drought stress tolerance (Frey et al., 1999; Thompson et al., 2000; Iuchi, et al., 2001; L. Xiong and J.-K. Zhu, unpublished data) suggests that ABA biosynthesis is coordinately controlled at multiple steps. Alternately, it may result from the positive regulation of ABA biosynthetic genes by ABA, because a limited initial increase in ABA biosynthesis from overexpressing a single ABA biosynthetic gene may result in a coordinately increased induction of other ABA biosynthetic genes (Xiong et al., 2002).

The mechanisms by which drought or salt stress upregulate ABA biosynthetic genes are not understood. Recent studies suggest that all of these genes (i.e., *ZEP*, *NCED*,

AAO3, and *MCSU*) are likely regulated through a common cascade that is Ca^{2+} dependent (L. Xiong and J.-K. Zhu, unpublished data) (Figure 5).

Transcriptional Activation of Stress-Responsive Genes

Molecular studies have identified many genes that are induced or upregulated by osmotic stress (Ingram and Bartel, 1996; Bray, 1997; Zhu et al., 1997). Gene expression profiling using cDNA microarrays or gene chips has identified many more genes that are regulated by cold, drought, or salt stress (Bohnert et al., 2001; Kawasaki et al., 2001; Seki et al., 2001). Although the signaling pathways responsible for the activation of these genes are largely unknown, transcriptional activation of some of the stress-responsive genes is understood to a great extent, owing to studies on a group of such genes represented by *RD29A* (also known as *COR78/LTI78*) (Figure 4). The promoters of this group of genes contain both the ABRE and the DRE/CRT (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997). Transcription factors belonging to the EREBP/AP2 family that bind to DRE/CRT were isolated and termed CBF1/DREB1B, CBF2/DREB1C, and CBF3/DREB1A (Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998; Medina et al., 1999). These transcription factor genes are induced early and transiently by cold stress, and they, in turn, activate the expression of target genes. Similar transcription factors DREB2A and DREB2B are activated by osmotic stress and may confer osmotic stress induction of target stress-responsive genes (Liu et al., 1998). Several basic leucine zipper (bZIP) transcription factors (named ABF/AREB) that can bind to ABRE and activate the expression of ABRE-driven reporter genes also have been isolated (Choi et al., 2000; Uno et al., 2000). *AREB1* and *AREB2* genes need ABA for full activation, since the activities of these transcription factors were reduced in the ABA-deficient mutant *aba2* and ABA-insensitive mutant *abi1-1*, but were enhanced in the ABA-hypersensitive *era1* mutant, probably due to ABA-dependent phosphorylation of the proteins (Uno et al., 2000).

The ability of the CBF/DREB1 transcription factors to activate the DRE/CRT class of stress-responsive genes was further demonstrated by the observation that overexpression or enhanced inducible expression of CBF/DREB1 could activate the target genes. Overexpression also increased tolerance of the transgenic plants to freezing, salt, or drought stress (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 2001), suggesting that regulation of the *CBF/DREB1* class of genes in plants is important for the development of stress tolerance. Early signaling components upstream of CBF/DREB1 may be subjected to specific ubiquitination-mediated degradation, as suggested by the molecular cloning of the Arabidopsis *HOS1* locus (Lee et al., 2001). *hos1* mutant plants show enhanced cold induction of stress-responsive genes,

but salt or ABA induction of these genes was not substantially altered (Ishitani et al., 1998). *HOS1* encodes a novel protein with a RING finger motif similar to those present in a group of IAP (inhibitor of apoptosis) proteins in animals that act as E3 ubiquitin ligases to target certain regulatory proteins for degradation. *HOS1* may perform a similar function in cold signal transduction (Figure 4) by targeting a positive regulator(s) of CBF/DREB1 expression for degradation, because the expression levels of the CBF/DREB1 genes in *hos1* are higher than those in wild-type plants under cold stress (Lee et al., 2001). Additionally, the nucleo-cytoplasmic partition of *HOS1* protein is regulated by cold. At normal growth temperatures, *HOS1* resides in the cytoplasm, but appears to relocate to the nucleus upon cold treatment, suggesting that *HOS1* may relay the cold signal to the nucleus to regulate the expression of *CBF/DREB1* genes (Lee et al., 2001).

The fact that some stress-responsive genes such as *RD22* do not have the typical DRE/CRT elements indicates that they may be activated through different mechanisms. A MYC transcription factor, *RD22BP1*, and a MYB transcription factor, *AtMYB2*, were shown to bind *cis*-elements in the *RD22* promoter and cooperatively activate *RD22* (Abe et al., 1997). In Arabidopsis, several putative two-component response regulators have Myb-like DNA binding motifs (Urao et al., 2000). Two of these proteins, *ARR1* and *ARR2*, were shown to be transcription factors capable of binding to specific *cis* DNA sequences and activating a reporter gene or genes for mitochondrial complex I (Sakai et al., 2000; Lohrmann et al., 2001). More recently, Hwang and Sheen (2001) presented experimental evidence that *ARR1* and *ARR2* are transcriptional activators that are positively regulated by the histidine phosphotransmitter (AHP) downstream of hybrid histidine kinase cytokinin receptors. AHP proteins are translocated into the nucleus from the cytosol in a cytokinin-dependent manner. It is unknown whether similar 'shortcut circuitries' involving pseudo-responsive regulators function in hyperosmolarity signaling. However, a variant of this type of short pathway in salt stress signaling is conceivable. A CDPK from the common ice plant, *MsCDPK1*, interacts with and phosphorylates *CSP1* in a Ca^{2+} -dependent manner (Patharkar and Cushman, 2000). The sequence of *CSP1* is similar to that of Arabidopsis *ARR1* and *ARR2*. Salt stress also stimulates the translocation of *MsCDPK1* to the nucleus, where *CSP1* is localized. Furthermore, *CSP1* can bind to the promoters of several stress-responsive genes (Patharkar and Cushman, 2000). Together with the study showing that activated *CDPK1* can induce stress-responsive gene expression (Sheen, 1996), these findings raise the possibility that some CDPKs regulate *CSP1*-like transcription factors upon activation by Ca^{2+} and consequently activate the expression of some stress-responsive genes.

In addition to the transcription factors that directly bind to the *cis*-elements in the promoters of stress-responsive genes, transcriptional activation needs additional cofactors that can also be important in determining the levels of gene

expression. When overexpressed in Arabidopsis and tobacco, the soybean gene *SCOF-1* (encodes a zinc-finger protein) can activate *COR* gene expression and increase freezing tolerance in nonacclimated transgenic plants, although the *SCOF-1* protein does not directly bind to either the DRE/CRT or the ABRE elements (Kim et al., 2001). *SCOF-1* interacts with another G-box binding bZIP protein, SGBF-1. SGBF-1 can activate ABRE-driven reporter gene expression in Arabidopsis leaf protoplasts. Thus, *SCOF-1* may regulate the activity of SGBF-1 as a transcription factor in inducing *COR* gene expression (Kim et al., 2001). In Arabidopsis, CBF1-mediated transcription may also require the transcriptional adaptor ADA and the histone acetyltransferase GCN5 (Stockinger et al., 2001). It is expected that gene mutations or altered activities in these components may affect low-temperature regulation of *COR* gene expression without affecting the expression of *CBF/DREB1* genes. Mutations such as the Arabidopsis *sfr6* (Knight et al., 1999) appear to fall into this category. The *sfr6* mutants show reduced expression of some *COR* genes, but the expression of *CBF/DREB1* genes is not affected (Knight et al., 1999).

Categorizing Stress Signaling Pathways: Outputs, Specificity, and Interactions

Many signal transduction processes occur when plants are challenged with environmental stresses. However, there has been no consensus for how to categorize these many signaling events. On the basis of the above discussion on the major signaling processes, we think that the signal transduction networks for cold, drought, and salt stress can be divided into three major signaling types (Figure 6): (I) osmotic/oxidative stress signaling that makes use of MAPK modules, (II) Ca^{2+} -dependent signaling that lead to the activation of *LEA*-type genes (such as the DRE/CRT class of genes), and (III) Ca^{2+} -dependent SOS signaling that regulates ion homeostasis. Type I signaling may contribute to the production of compatible osmolytes and antioxidants, and may also relate to cell cycle regulation under osmotic stress. Representative mutants that might be affected in this signaling branch include the freezing-tolerant mutant *eskimo1* (*esk1*) and the salt-tolerant mutant *photoautotrophic salt tolerance 1* (*pst1*). *esk1* accumulates increased amounts of proline and soluble sugars, but the expression of the DRE/CRT class of genes is unaffected (Xin and Browse, 1998). The *pst1* mutant shows increased ROS scavenging capacity but appears unaltered in the accumulation of Na^+ (Tsugane et al., 1999). Type II signaling leads to the activation of the DRE/CRT class and other types of *LEA*-like genes, and is the most extensively studied. Mutants defective in this signaling type include some of the *cos*, *hos*, and *los* mutants isolated in an *RD29A-LUC* reporter-facilitated genetic screen (Ishitani et al., 1997). Some of these mutations (e.g., *fry1*, *hos1*, *los5*, *los6*, and *sad1*) have been cloned. Their roles in stress signaling were dis-

cussed in the preceding sections. Type III signaling appears to be relatively specific for the ionic aspect of salt stress (Figure 6). Targets of this type of signaling are ion transporters that control ion homeostasis under salt stress. The *sos* mutants (*sos3*, *sos2*, and *sos1*) fall into this category. These mutants are hypersensitive to salt stress, but activation of the DRE/CRT class of genes is unchanged in them (Zhu et al., 1998). In addition, salt-induced accumulation of the compatible osmolyte proline was not reduced but rather was enhanced in the *sos* mutants (Liu and Zhu, 1997b). The enhanced proline production represents a compensatory response likely triggered by reduced salt tolerance in the mutants. Besides these major signaling routes, some additional pathways also exist, as discussed earlier.

One important issue regarding various stress signal transduction pathways is their specificities with respect to the input stimuli. The specificity and interaction between pathways have been addressed explicitly (Knight and Knight, 2001). As discussed before, each of the stress conditions (i.e., cold, drought, and high salinity) has more than one attribute. If two stress conditions have a common attribute (for example, hyperosmotic stress for drought and salinity), then the signaling arising from this common attribute might not be specific for either of the stress conditions. Additionally, it is important to distinguish the particular pathways when signaling specificity is considered. Interaction among these three signaling types (Figure 6) is not extensive, as evidenced by the lack of mutants defective in more than one of the signaling types and by the results from additional transgenic studies discussed above (e.g., Kovtun et al., 2000). For instance, osmotic stress activation of the MAPKs SA-induced protein kinase and HOSAK in tobacco is independent of ABA and is not affected by the *sos3* mutation (Hoyos and Zhang, 2000). Likewise, although both drought and salt stress result in a transient increase in cytosolic Ca^{2+} , drought stress does not appear to activate the SOS pathway. It is possible that these different stresses have different Ca^{2+} signatures that could be decoded by their respective Ca^{2+} sensors. Specific Ca^{2+} oscillations in guard cells in the regulation of stomatal movements have been reported (Allen et al., 2001). Limited interaction between some of the different signaling pathways may be due to overlap in the detection range of Ca^{2+} sensors, particularly with respect to recurrent Ca^{2+} transients, which result from multiple rounds of stimulation by secondary signal molecules (Figure 2). Under certain circumstances, e.g., when a signaling component is overexpressed or ectopically expressed, unnatural interactions among the different signaling pathways may occur. One of the causes of this 'gain-of-function' effect is the alteration of either the original subcellular localization or the dosage of the signaling molecules. Therefore, caution should be exercised when inferring the in vivo function or epistasis of genes from phenotypes caused by overexpression or dominant mutations.

In contrast to the limited interaction among the major different signaling routes (Figure 6), interaction within a signal-

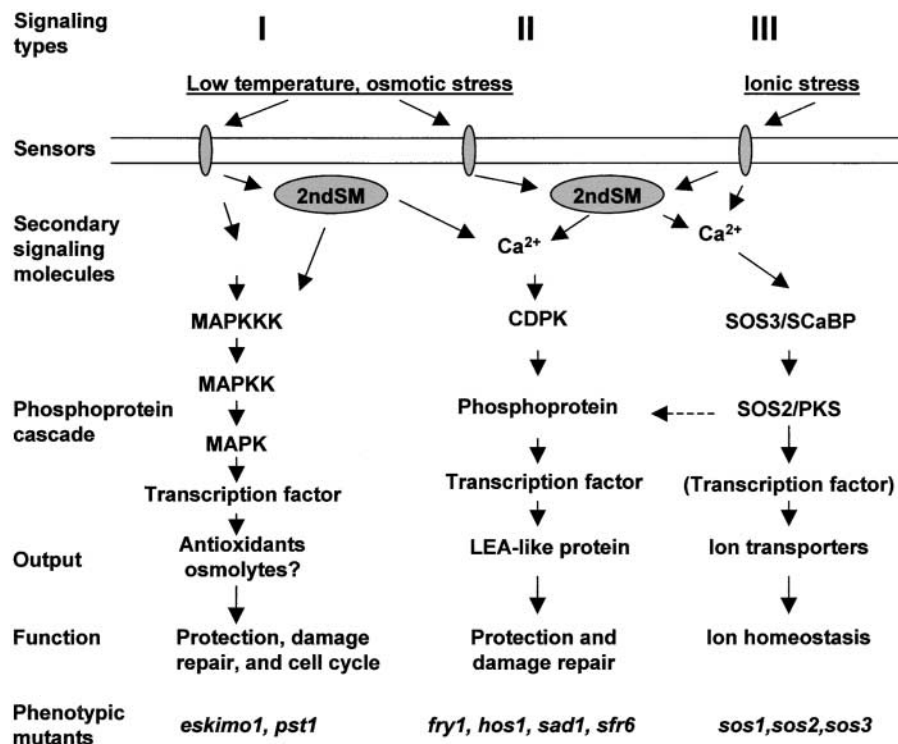


Figure 6. Major Types of Signaling for Plants during Cold, Drought, and Salt Stress.

Representative cascades, outputs, biological functions, and examples of mutants with phenotypes indicative of defects in the respective biological functions are shown. Type I signaling involves the generation of ROS scavenging enzymes and antioxidant compounds as well as osmolytes. The involvement of a MAPK pathway in the production of osmolytes in plants has not been demonstrated experimentally. Under osmotic stress, altered MAPK signaling may contribute to changed cell cycle regulation and growth retardation. Type II signaling involves the production of stress-responsive proteins mostly of undefined functions. Pathways within Type II signaling are shown in Figure 4. Type III signaling involves the SOS pathway which is specific to ionic stress. Signaling events for homologs of SOS3 (SCaBP) and SOS2 (PKS) are tentatively grouped with SOS3 and SOS2, yet these SCaBP-PKS pathways are not necessarily related to ion homeostasis. Connections between different types of signaling events are indicated with dashed lines. Arrows indicate the direction of signal flux. Primary sensors are shown to be localized in the membrane. Receptors for secondary signaling molecules (2ndSM) are not shown.

ing type can be fairly extensive. This is best illustrated by the study of *RD29A-LUC* induction, as revealed by mutational analysis of the pathways (Ishitani et al., 1997) and further characterization of several mutants, as discussed above (Figure 4). Additional discussion on pathway interaction for the activation of *LEA*-type genes can be found in recent reviews (Shinozaki and Yamaguchi-Shinozaki, 2000; Knight and Knight, 2001). Similarly, interaction between MAPK pathways is also common, as discussed in previous sections.

CONCLUDING REMARKS

Although this review of abiotic stress signal transduction in plants covers only a portion of the relevant studies, it is evi-

dent that the subject is very complex and that exciting progress is being made. Genetic approaches are important tools for analyzing complex processes such as stress signal transduction. Conventional genetic screens based on stress injury or tolerance phenotypes have been applied with success (Zhu, 2000). However, such screens may not be able to identify all components in the signaling cascades due to functional redundancy of the pathways in the control of plant stress tolerance (Xiong and Zhu, 2001) (Figures 4 and 6). The accessibility of the Arabidopsis genome and various reverse genetics strategies for generating knockout mutants should lead to the identification of many more signaling components and a clearer picture of abiotic stress signaling networks. Molecular screens such as the one using the *RD29A-LUC* transgene as a reporter (Ishitani et al., 1997) are beginning to reveal novel signaling determinants (Figure 4). Similar approaches may prove useful for the study of

other pathways, such as osmolarity sensing (type I signaling; Figure 6). Adoption of forward and reverse genetic approaches by more researchers in this field will certainly expedite our understanding of stress signaling mechanisms in plants.

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